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13. ABSTRACT (Maximum 200 words) Our hypothesis is that antibodies, covalently bonded to inert membranes, will be charged, depending on the pH of the ambient electrolyte. A positive surface charge is a possible situation in low pH bathing solution. At low bathing concentrations of a pilot salt, such as sodium tetraphenylborate, sodium ions will be excluded from the membrane but anions will try to penetrate. To help the penetration we give the system a small driving force: unequal concentrations of pilot salt on the two sides. We expect a surface-charge-controlled membrane potential to arise initially. When antigen is added, a change in surface charge upon surface immunoreaction may cause an abrupt change in the membrane potential that we could use as an analytical signal. When there is no surface site charge, or when there is too high concentration of bathing electrolytes, there is a collapse of Donnan exclusion and salts simply diffuse through the membrane without regard for the surface charge barrier. This report contains the results of this investigation.				
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ELECTROCHEMICAL DONNAN EFFECT BIOSENSORS

FINAL REPORT

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JUNE 30, 1990

U. S. ARMY RESEARCH OFFICE

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1. FOREWORD:

This program began with the idea that underlies the famous Esin-Markov Effect in modern interfacial electrochemistry as described by R. Parsons, "Equilibrium Properties of the Electrified Interface", in "Modern Aspects of Electrochemistry" (J. O'M. Bockris and B. E. Conway, eds.) , Butterworths Sci. Pubs., London, 1954, pp 103-179. If an interface between conducting phases will adsorb a constant amount of a charged material, even when the interfacial potential difference is changed, then spontaneous potential changes can be related to compensating charges of dissolved species in one phase. This proposition was tested using strongly adsorbing iodide ions on mercury, while varying the concentration of soluble iodide salt in one phase (the water).

The theme was used to interpret the first pH sensing ISFET in the first plenary lecture of the first Conference of Chemically Sensitive Semiconductor Devices: R. P. Buck, "Potential Generating Processes at Interfaces: from Electrolyte/Metal and Electrolyte/Membrane to Electrolyte/Semiconductor", in "Theory, Design, and Biomedical Applications of Solid State Chemical Sensors", (P. W. Cheung, D. G. Fleming, W. H. Ko, and M. R. Neuman, eds.), CRC Press, Boca Raton, Fla. 1978 pp 3-40. It was later a theme for biosensors and proposed and explained at the 1985 IUPAC Congress in Manchester, England in 1985 and is printed in R. P. Buck, "Biosensors Based on Reversible Reactions at Blocked and Unblocked Electrodes", IUPAC 30th International Congress, Symposium on New Electrochemical Sensors, Proceedings Volume, (J. Alberly, ed.), Royal Society of Chemistry, J. Chem. Soc., Faraday Trans. I, 1986, 82, 1169-1178.

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4. REPORT:

A. STATEMENT OF THE PROBLEM STUDIED

THE IDEAS OF DONNAN EXCLUSION AND DONNAN FAILURE AND SENSORS BASED ON CHARGE CANCELLATION

Our hypothesis is that antibodies, covalently bonded to inert membranes, will be charged, depending on the pH of the ambient electrolyte. A positive surface charge, shown in Fig. 1 (left) is a possible situation in low pH bathing solution. At low bathing concentrations of a pilot salt, such as sodium tetraphenylborate, sodium ions will be excluded from the membrane but anions will try to penetrate. To help the penetration we give the system a small driving force: unequal concentrations of pilot salt on the two sides. We expect a surface-charge-controlled membrane potential to arise initially. When antigen is added, a change in surface charge upon surface immunoreaction may cause an abrupt change in the membrane potential that we could use as an analytical signal. When there is no surface site charge, or when there is too high concentration of bathing electrolytes, there is a collapse of Donnan exclusion (Fig. 1 right) and salts simply diffuse through the membrane without regard for the surface charge barrier.

The choice of pilot salt should depend on the surface charge. We expect the largest signal to be developed by using a salt whose larger (lower mobility ion) has the same sign as surface charge. Expected surface charge sign, and pilot (or probe) salts are in Fig. 2. We expect a sensor to function only when the aqueous solution is very dilute so interfacial potential differences change maximally with soluble, charge-compensating salt, e.g. 59 mV/decade. At constant bathing salt solution with varying antibody (or antigen depending on which is immobilized on the surface) charge cancellation should be accompanied by a potential change as well.

B. SUMMARY OF THE MOST IMPORTANT RESULTS

OUR ORIGINAL WORK PLAN:

- a. Select and characterize site contents of cellulosic and Millipore Affinity Immobilon polyvinylidene polymers as substrates for the electrochemical interface.
- b. Attach IgG to the surface of a cellulosic or poly(vinylidene difluoride) polymer membrane.
- c. Select aqueous electrolytes with two very hydrophilic or very hydrophobic ions; one hydrophilic cation, hydrophobic anion;

one hydrophobic cation, hydrophilic anion. Hydrophilic = water loving, water 'favoring'; hydrophobic = oil loving, membrane favoring.

d. Determine whether buffers must be used, how low the concentrations must be, and whether other salts are tolerated, or whether they simply destroy the measured potential effect.

e. At fixed selected bathing salt concentration, determine how measured potentials vary with added anti IgG.

f. Measure the interfacial impedance to demonstrate whether or not an interfacial antigen-antibody reaction is occurring, and whether it is accompanied by a change in surface resistance.

g. Measure the binding of antigen-antibodies at a silver electrode using surface enhanced Raman spectroscopy.

Results for a. of Workplan

Dr. Sakura characterized a long series of membranes according to their fixed-site concentrations and charges. She used the classical Meyer-Bernfeld plots (membrane potential vs concentration of uni-univalent salt on one side while holding the concentration on the second side at a constant factor of 1/10). At high bathing concentrations, the membrane passes salt from high to low bathing solutions, i.e. the Donnan failure occurs and measured potentials depend only on mobilities of the permeating ions of the salt. At lower bathing concentrations, the membrane potential increases, absolutely, as the membrane becomes permselective to cations only. The Meyer-Bernfeld plot allows you to calculate the fixed-site charge in the membrane. It is generally negative and 10^{-5} to 10^{-4} M for cellulosic membranes. These values tell us what bathing concentrations to use for our biosensors. Obviously, in agreement with intuition, they must be very dilute solutions.

Dr. Sakura repeated the measurements, in the Donnan failure concentration range using all of the common +1 cation chloride salts including tetraalkyl ammonium salts up to tetrahexyl ammonium. The correlation of diffusion potentials with transference numbers and with ion sizes was clear. Although these membranes are not optimal as supports for immunochemicals, it seems necessary to do these measurements to get good background data on the charge sign and charge concentration in the membranes. These numbers tell us how much charge on the immunochemical surface layer is required to be larger or smaller the preexisting membrane charge. If we are to modulate ion transport by the immunochemical reaction layer, we must have more charge (ion exchange capacity) in the surface layer than is already present in the membrane itself. This number for the surface layer is determined by extent of surface coverage, and by the pH of the aqueous bathing solution.

Results for b. of Workplan

Attachment of antibodies to cellulosic membranes proved to be too difficult for us. We found that a commercial product Immobilon polyvinylidene difluoride polymers are made for antibody attachment, according to a recipe. Dr Sakura, who was expected to do more work, was married to an American, returned to Japan for long periods and was not able to help in the later phases of the work.

Results for c. of Workplan

We had no difficulty attaching human IgG to Immobilon membranes and selected 'pilot ion' electrolytes for trials: NaCl (all hydrophilic); TEAClO₄, (tetraethylammonium perchlorate both hydrophobic); NaTPB (sodium tetraphenylborate, anion hydrophobic); and TEACl (tetraethylammonium chloride, cation hydrophobic). When anti-human IgG was added to the solutions of pH 4 (no buffer added) and pH 7, sodium chloride, with its compatible ion mobilities did not show a potential difference in any of the tests. NaTPB did show responses that were superior (e.g. larger) to any of the other salts.

Results for d. of Workplan

Experiments were conducted in which NaCl was added to the 0.0001M NaTPB bathing solutions, at increasing concentrations up to values approaching body fluids (e.g. 0.15 M). Responses were not influenced at 0.0001 M, but were barely visible at 0.001 M, but disappeared at higher quantities of added NaCl. This result limited buffers are pH 4 and pH 7 to extreme dilution, e.g. 0.0001 M in buffer components.

Results for e. of Workplan

Studies of the commercially available Millipore Immobilon affinity membranes have continued using human IgG. Edith Grabbe and the undergraduates concentrated on this membrane system. The generation of a potential difference had been noted for membranes coated on both sides with IgG when asymmetrically bathed in cells of the type:

junction	high conc. side	membrane	low conc. side	junction
reference	.0001 M NaTPB		.00001 M NaTPB	reference
electrode				electrode

where TPB = tetraphenylborate. The membrane potentials of cells with untreated membranes were stable, but those for IgG-coated membranes were relatively unstable, at least not reproducible enough for reliable sensor applications. Edith Grabbe systematically prepared membranes with different surface loadings and different capping solutions (to bind to

unreacted surface sites and prevent general adsorption). We could not discover a recipe that would produce uniform, reproducible surface layers of IgG. We have subsequently found that part of the apparent lack of reproducibility was our lack of appreciation of how to remove bonded anti-IgG. We had assumed the processes were rapid and reversible and that washing in water (buffered) would return an IgG-coated membrane back to its initial state. This is not the case! The experiment may have actually worked very well. Results were reported in an earlier progress report.

Results for f. of Workplan

Potentiometric techniques were replaced by impedance spectroscopic measurements. We suggested this alternative measurement in our original proposal. These experiments contain more information about the membrane/solution interface e.g. resistance and capacitance, and may result in enhanced signals. They are not passive measurements, but the perturbation of the membrane and surface film is small, typically 100 mV.

In impedance experiments, the cell is the type:

Ag/AgCl	high conc. side	membrane	low conc. side	Ag/AgCl
reference	.0001 M pilot		.00001 M pilot	reference
electrode	salt		salt	electrode
	.001 M buffer		.001 M buffer	
	.00001 M NaCl		.00001 M NaCl	

where Ag/AgCl = silver/silver chloride. The experiment consists of applying a voltage, alternating in polarity with varying frequency, between the two flag-shaped reference electrodes and calculating the real and imaginary components of the impedance from the current generated. The voltage bias used in these experiments is 100 mV and the frequency varies between 65500 and .5 Hz.

Membrane coverage of IgG was determined by calculating the difference in UV absorbance at 280 nm of buffered IgG solutions before and after reaction with the membranes. Uptake of IgG to form surface coatings was found to be very reproducible, and averaged 25 µg/ 13 mm disk, which is about twice what Millipore reports. However, this difference can be explained by noting that these measurements were taken before capping of excess surface sites and before washing with TWEEN 20. Some protein would be expected to be lost in these preparation steps.

The simple series combination of solution resistance and two parallel circuit elements (for bulk transport and for surface resistance or activation) are illustrated in Fig. 3. The expected impedance plane plots for the circuit is illustrated schematically in Fig. 4. Frequency of each point increases from right to left. Surface effects occur at the lower frequencies (right hand semicircle) and bulk transport properties

characterize the high frequency (left hand) semicircle. The first impedance experiments conducted used similar conditions to those of the potentiometric tests: pH 4 with no buffering and NaTPB for pilot salt. These tests revealed a large geometric semicircle due to the bulk membrane impedance, measuring .2 M Ω (resistance) and near 5 pF (capacitance). The width of this semicircle, e. g. the resistance, decreased linearly with addition of antigen, but the change was not immunospecific, because it occurred with addition of anti-IgA as well. A typical impedance diagram is given in Fig. 5. Soaking of the membrane, resulting in some desorption of IgG from the surface, or simply wetting of the membrane, may be responsible for this decrease in resistance. The membranes displayed only small surface related semicircles at this pH. These did not change in any reproducible way with addition of antigen. Although sometimes, decreases in resistance were seen with addition of antigen, it was not immunospecific. We did not expect IgG would be very active at this pH.

Since the membranes did display a large resistance, it was thought that buffering solutions could be employed to raise the pH to a more biologically active level, without causing Donnan failure. Phosphate buffers were first used with ionic strength of .01 M. These proved to be too concentrated, because the resistance of the membrane dropped to zero by Donnan failure. Dilute buffers of 1 mM ionic strength had enough buffer capacity to raise the pH, but not overload the membranes' ion exclusion capacity. The membranes, in these solutions, show smaller bulk resistances, probably by some leakage of buffer. Also, IgG is uncharged at this pH and would not contribute to Donnan exclusion. The bulk resistance decreases linearly with antigen addition and, in this case, the response is immunospecific. FAB specific anti-IgG displayed excellent responses that were more than four times the sensitivity of Fc specific anti-IgG and only minimal decreases for anti-IgA. The FAB specific antigen would be expected to yield a greater response since it has two active binding sites for each IgG molecule.

The most interesting feature of the membranes at this pH is the growth of a surface semicircle from a large overlapping series of resistances as IgG-specific antigen is added. The broad surface feature seen before addition of antigen is probably due to inhomogeneities in coating of IgG on the surface or in channels in the membranes. When IgG-specific antigen is added to the solution, this feature grows into a well-defined semicircle, whose resistance remains fairly constant but whose capacitance increases with further addition of antigen until a one-to-one ratio of antigen to antibody is attained.

Other studies of these membranes included varying the methods of preparation, including decreasing the IgG concentration in the coupling solution and varying the method of capping the excess active sites on the membrane surface. None of these changes produced more reliable or more sensitive membranes, so our standard methods of preparation were continued.

SURFACE IMPEDANCES AS A FUNCTION OF SOLUTION pH AND PILOT SALT

In the following impedance experiments, the cell given above used in sequence:

for pH 5	0.001 M NaOAc and HOAc
for pH 7	0.001 M Na_2HPO_4 and NaHPO_4
for pH 9	0.001 M NaHCO_3 and Na_2CO_3

Pilot salts were: sodium tetraphenylborate, tetraethylammonium perchlorate, and tetrabutylammonium chloride.

Studies included cell impedance responses to other pH values, keeping the solutions buffered with acetate, phosphate, or carbonate buffers. The pilot salts used in the experiments were also varied. The pilot salt, sodium TPB, used in the most of our reported studies, has a cation which is much smaller and more hydrophilic than the anion. Tetraethylammonium perchlorate, with anion and cation of nearly equal mobilities, is quite hydrophobic. Tetrabutylammonium chloride has a large hydrophobic cation and small hydrophilic anion, just the opposite of sodium tetraphenylborate. These salts have also been exposed to a series of pH buffer including 5, 7 and 9. Studies have been conducted using similar concentrations of NaCl, but this salt passed too easily through the membrane, and showed no bulk resistance changes when the membrane was coated with IgG.

A summary of the real impedances (pure resistances in kilohms) of the Immobilon membrane with its surrounding solutions, are given in the attached table. The second column is the impedance of the bathing electrolytes between the two low-impedance source/sensing electrodes. The electrodes, per se, are so large that they contribute less than 1000 ohms to the total. When a clean membrane is interposed, the resistance hardly increases at all (column 3). The IgG-coated membranes appear to contribute additional resistances depending on the probe salt (and pH) used to make the resistance measurements. This effect is desired since it tells us that some salts penetrate the film more easily than others. Sodium tetraphenylborate gives the largest incremental resistance of the pilot salts tested. It is not surprising that the incremental resistance depends so much on the salt, and on pH.

This result may justify our hypothesis that the surface resistance barrier is mainly determined by surface charge, but also depends on ion mobility in the surface packing. The two entries for NaTPB show this point. When the membrane is positive, TPB⁻ apparently has difficulty in passing the packed surface. The response at pH 9 when the surface is negative shows a much larger resistance increment, presumably from the negative surface charge. The split between resistance of the bulk semicircle and the surface semicircle is not always visible. The data in the table are simply combinations of the semicircle resistances to give total resistances.

ESSENTIAL WORK THAT SHOULD HAVE BEEN DONE

In addition to "holes" in the data, we need some measures of the activity of surfaces layers rather than surface coverage. It is not clear that low coverage membranes are useful if the IgG becomes inactive (denatured). Use of radiotracers or fluorescent tags to distinguish activity from coverage should have been considered early. Coverage can be found (in addition to the spectroscopic monitoring method already used) from radio-IgG bound. Then radio-anti-IgG would be used to see active binding. Impedance observations of the surface resistance semicircle, to determine what conditions make it appear, should have been done.

We should have realized that immobilization of active antibodies (or antigens) on membrane surfaces requires expertise. We needed a real Immunochemist on this project with the patience to make and test many systems until a reproducible membrane and layer were made. Then we should have found out that antibody reactions are not quickly reversible. Some of our negative results probably occurred because we did not wait long enough to reverse the system and return it to its initial condition.

PRIVATELY REPORTED SUPPORT FOR THE FEASIBILITY OF OUR ORIGINAL PROPOSAL

Last Fall, Professor Karl Cammann showed me his new 8 million Deutsche Mark biosensor facility at the Wilhelms University of Westfalia in Munster. We have often talked about sensors based on blocking, and on charge cancellation effects. He had independently studied similar systems with the same principal being tested. He uses immunochemists to prepare active antibody layers on pNa glass (sodium-sensing glass electrodes). The electrode responses to sodium ion are affected by the antigen-antibody reaction, but only when the concentrations of sodium ions are varied in the very low concentration range (less than 10^{-4} M). At a selected very low Na level, the electrode responds to the changing antigen level, in accordance with our results.

OTHER RELATED STUDIES SUCCESSFULLY COMPLETED

Edith Grabbe studied the binding of IgG on silver, and the binding of anti IgG on silver. This spectroscopic work has caused some interest around the world, based on her first paper. The indications of immunochemical reaction between the two species is, however, not so very clear. There are two manuscripts from the latter work that were submitted, and are being rewritten.

Junguo Zhao studied the electrocatalytic properties of NMPTCNQ (N-methylphenazinium tetracyanoquinodimide) on metal and carbon electrodes. We then realized that the material is, itself, a metal (or has metal-like electrochemical properties). We studied the conductivity properties of the powder in plasticized slurries, and as a support for biosensors, using an ethanol

sensor as an example.

Mrs. Sakura Chapman continued this study, on a fundamental level, by measuring the electrocatalytic properties of the NMPTCNQ and TTF (tetrathiafulvalene)TCNQ electron promoting surface layers. These are promoters for use in amperometric sensors. She has prepared two manuscripts on the catalytic cycles (turn overs for the glucose-glucose oxidase couple and the ethanol-alcohol dehydrogenase system.

C. List of all Publications and Technical Reports and Oral Presentations Supported by CONTRACT NUMBER DAAL03-86-K-0055

Work of E. S. Grabbe:

E. S. Grabbe and R. P. Buck, "Evidence for a Conformational Change with Potential of Adsorbed Anti-IgG Alkaline Phosphatase Conjugate at the Silver Electrode Interface Using SERS" (Submitted to J. Electroanal. Chem.,).

E. S. Grabbe and R. P. Buck, "Surface Enhanced Raman Spectroscopic Investigation of Human Immunoglobulin G Adsorbed on a Silver Electrode", J. A. C. S., 111 (1989) 8362-8366.

Oral Presentation from the 22nd Middle Atlantic Regional Meeting of the American Chemical Society, seminar entitled "Donnan Effect Biosensors for the Detection of Immunochemicals," May 1988.

Work of J.-G. Zhao:

Junguo Zhao and R. P. Buck, "A Nearly All-Solid-State Amperometric Ethanol Sensor", Sensors and Actuators, (submitted January 1989).

Junguo Zhao and R. P. Buck, "Electrical Properties of NMPTCNQ Suspensions in A Low Dielectric Permittivity Plasticizer", J. Electrochem. Soc. 137 (1990) 2431-2437.

Junguo Zhao and R. P. Buck, "Electrical Properties of Inert Polymer Films Doped With Electrolytes", J. Electrochem. Soc., 135, (1988), 609-615.

Junguo Zhao and R. P. Buck, "Influence of Contact Materials on Electric Properties of Single Crystal AgBr", J. Electrochem. Soc., 136 (1989) 3342-3351. [Work done on the previous ARO Contract DAAG-29-84-K-0132].

Work of R. P. Buck:

R. P. Buck, "Steady State Diffusion-Migration Potential Differences in Mixed Conductor Polymer Films and Thin Layer Cells", J. Electroanal. Chem., 271 (1989) 1-14.

R. P. Buck, "General Voltage Step Responses and Impedances of Mixed Conductor Films and Diodes: Metal-Contact Cells With Mobile Anions or Cations", J. Phys. Chem., 93 (1989) 6212-6219.

R. P. Buck, "Coupled Electron Hopping-Anion Displacement in Plane-Sheet, Fixed-Site Polymer Membranes", J. Electroanal. Chem., 258 (1989) 1-12. [Work done on the previous ARO Contract DAAG-29-84-K-0132].

R. P. Buck, "Comparison of Steady State Electrical Properties for Two Counterion and Electron-Counterion Membranes", J. Phys. Chem., 92 (1988) 6445-6451. [Work done on the previous ARO Contract DAAG-29-84-K-0132].

R. P. Buck, "Diffusion-Migration Impedances for Finite, One-Dimensional Transport in Thin Layer and Membrane Cells, Part II: Mixed Conduction Cases: Os(III)/Os(II)ClO₄ Polymer Membranes Including Steady State I-V Responses", J. Electroanal. Chem., 219, (1987), 23-48. [Work done on the previous ARO Contract DAAG-29-84-K-0132].

D. List of All Participating Scientific Personnel and Advanced Degrees Earned While Employed on the Project.

Scientific personnel supported by this project during the entire period:

SENIOR PERSONNEL:

Dr. Sachiko Sakura, Visiting Professor from Fukuyama University, Fukuyama, Japan

GRADUATE STUDENTS:

Edith S. Grabbe
Michael L. Iglehart
Jun-guo Zhao
Thomas R. Berube

UNDERGRADUATE STUDENTS

Michael Fitzsimons
Matthew Wills
E. Yolanda Morgan
Lisa Roof

PhD Degrees Awarded - Title - (Current Address):

Michael L. Iglehart - March 1988, ION TRANSPORT PROPERTIES

OF NEUTRAL CARRIER-BASED, PLASTICIZED POLY(VINYL CHLORIDE)
MEMBRANES, (BASF, 1419 Biddle Avenue, Wyandotte, MI, 48192).

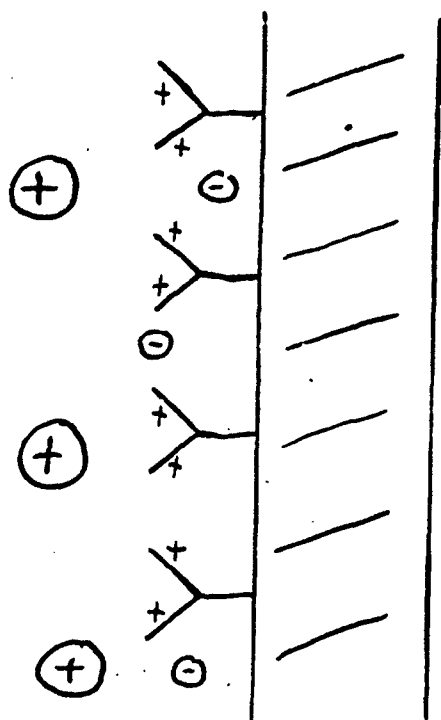
Edith S. Grabbe - January 1989, A SURFACE ENHANCED RAMAN
SPECTROGRAPHIC STUDY OF ANTIBODY/ANTIGEN BINDING ON SILVER
ELECTRODES, (NIST, A-361 Chemistry Building, Gaithersburg,
MD., 20988).

Jun-guo Zhao - March 1989, ELECTROCHEMICAL STUDIES OF MIXED
CONDUCTORS AND BIOSENSORS, (PostDoc position, Chemistry
Dep't. Duke University, Durham, N. C. 27706).

Thomas R. Berube - April 1989, RESPONSE OF ION-SELECTIVE
ELECTRODES TO STEPS IN ION CONCENTRATIONS, (Ethyl Technical
Center, PO BOX 14799, Baton Rouge, LA 70898).

DONNAN EXCLUSION

$10^{-4} M / 10^{-5} M$

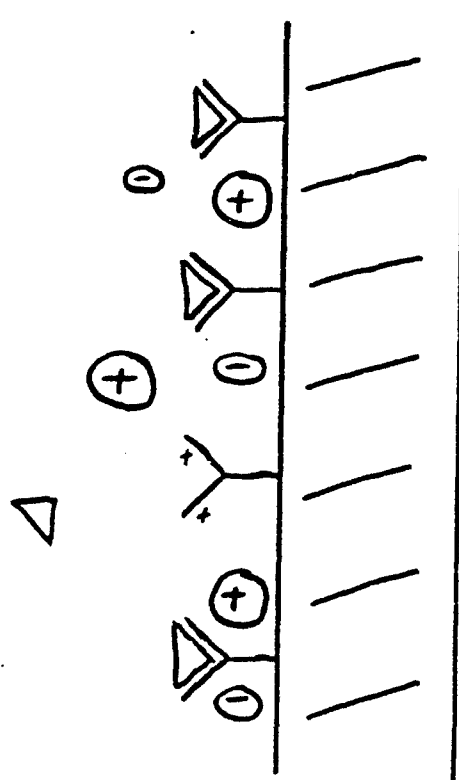


⊖ ANION →

⊕ CATION ↪

DONNAN FAILURE

$10^{-4} M / 10^{-5} M$

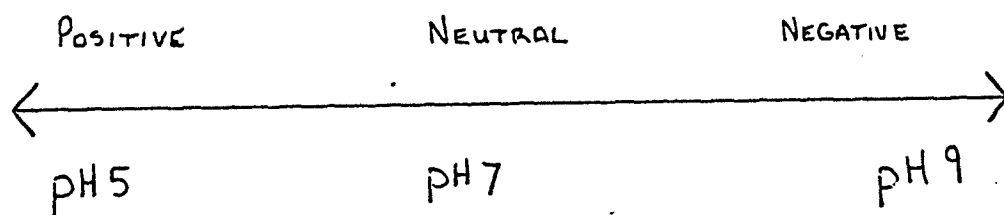


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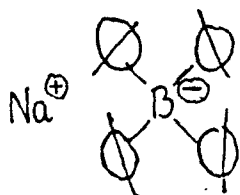
Fig1

ANTIBODY CHARGE

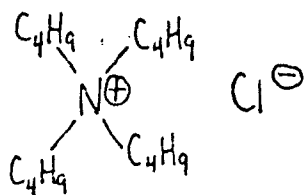


PROBE SALTS

SODIUM TETRAPHENYLBORATE NaTPB



TETRAETHYLAMMONIUM CHLORIDE TBACl



TETRAETHYLAMMONIUM PERCHLORATE TEAPClO₄

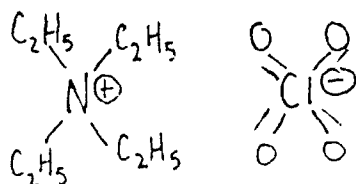
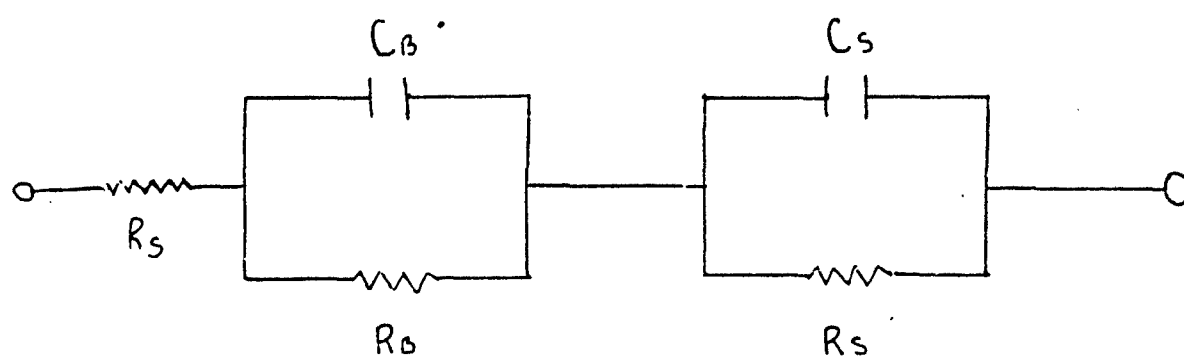


Fig 2

SOLUTION - MEMBRANE INTERFACE

EQUIVALENT RC CIRCUIT



SOLUTION

BULK

SURFACE
ACTIVATION
OVERPOTENTIAL

Fig 3

IDEAL IMPEDANCE PLOT

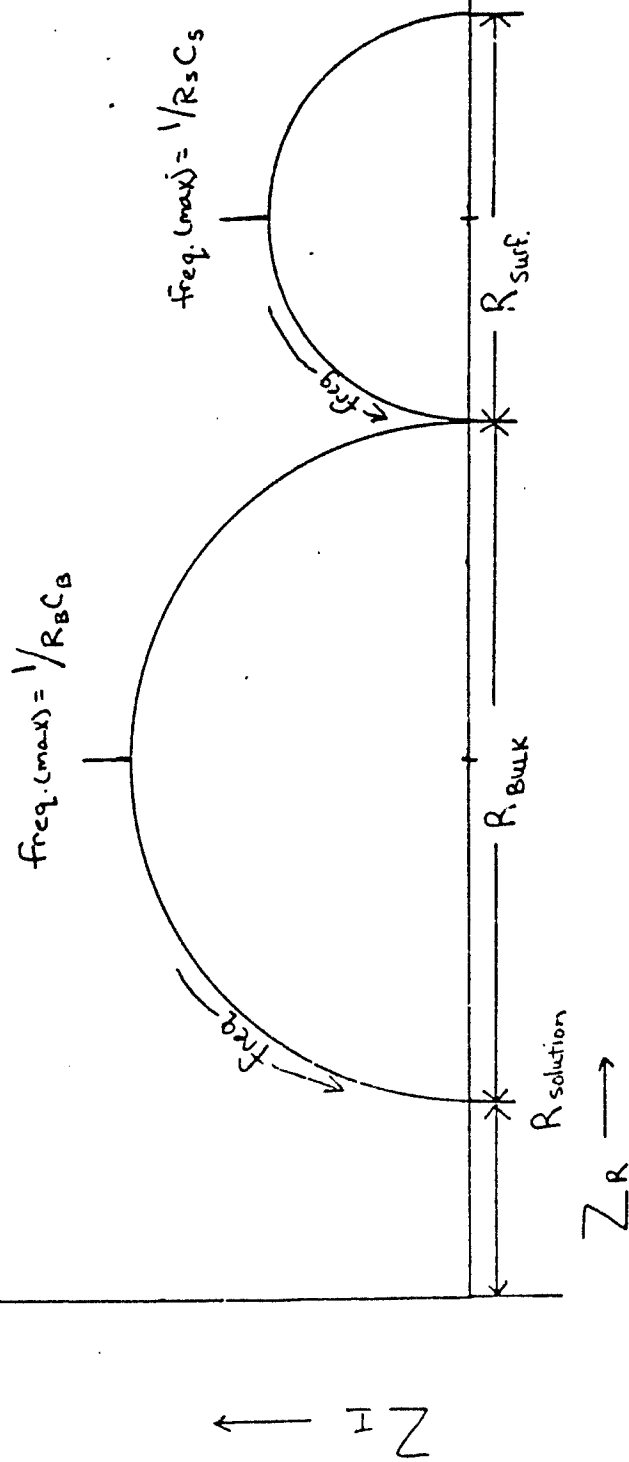


Fig. 4

IMPEDANCE PLANE PLOT

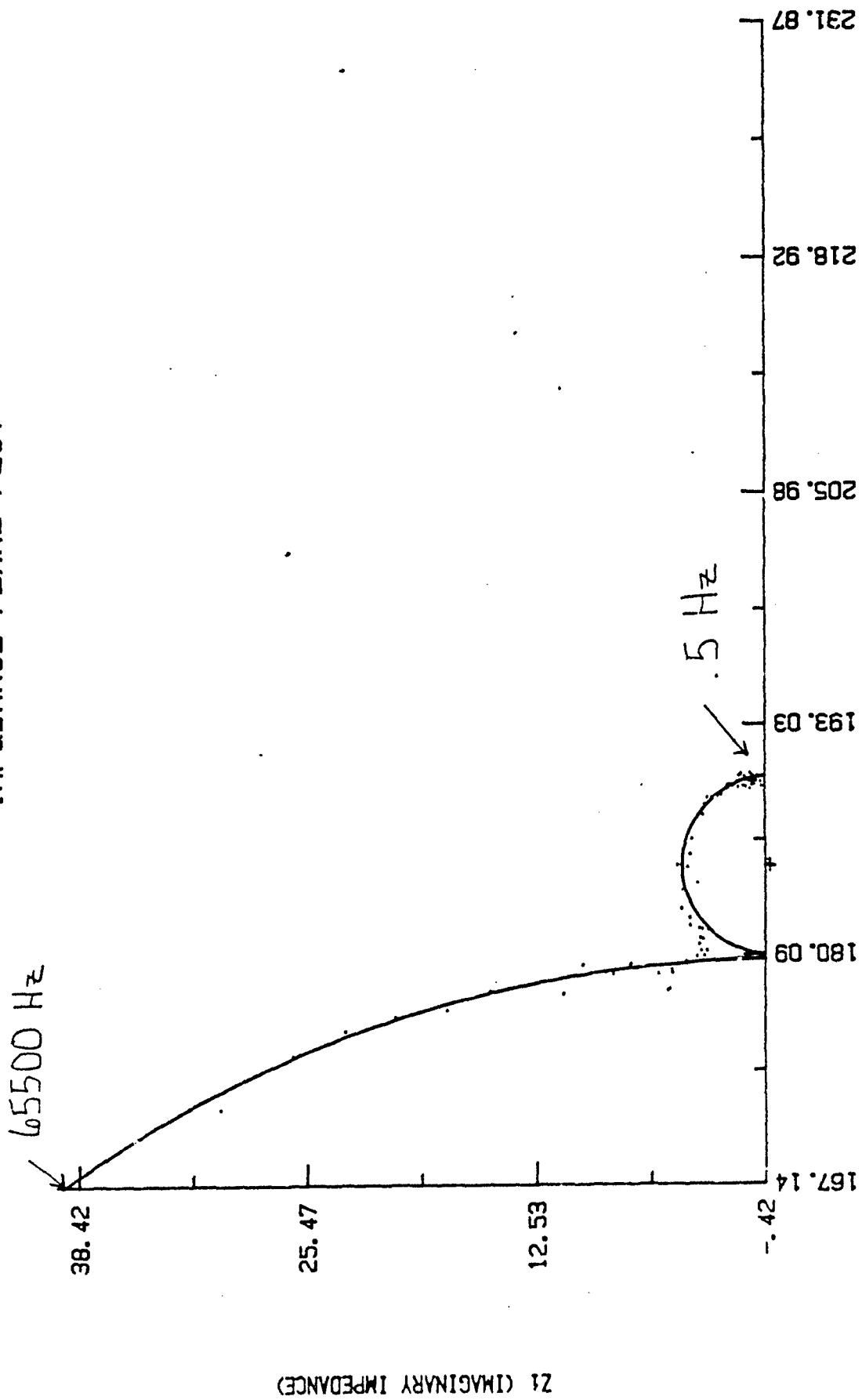


Fig 5

Zr (REAL IMPEDANCE)

Z1 (IMAGINARY IMPEDANCE)

SALT	pH	ONLY	BLANK MEMBRANE	CAPPED MEMBRANE	BOUND MEMBRANE
NaTPB	pH5	125.1	123.5	167.3	244.7
NaTPB	pH7	233.4	217.6	215.0	—
NaTPB	pH9	72.3	—	83.0	348.2
TEAClO ₄	pH5	124.7	116.5	171.5	206.0
TEAClO ₄	pH7	207.0	196.4	192.2	261.7
TEAClO ₄	pH9	—	—	96.5	—
TBACl	pH5	112.8	117.9	127.6	220.4
TBACl	pH7	242.3	253.0	250.0	236.9
TBACl	pH9	84.2	86.8	77.8	—